

# EVALUATING PROLIFERATION AND DIFFERENTIATION MARKERS IN DEVELOPING BRUNNER'S GLANDS

by

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Brunner's glands are located in the submucosa of the duodenum and are composed of secretory, glandular epithelium. Secretions from Brunner's glands contain bicarbonate, growth factors, and mucin glycoproteins. These secretions lubricate the duodenal mucosa and neutralize highly acidic chyme from the stomach as it enters the duodenum. Without this gland, highly acidic chyme from the stomach may cause ulceration. Adenomas of Brunner's glands are known to occur in humans and can result in potentially life threatening complications. Research on the development of Brunner's glands has largely been overlooked; it is still unclear how pathologies, such as adenomas of the glands, arise.

In order to understand the etiology of Brunner's gland pathologies, it is important to first understand the molecular mechanisms behind Brunner's gland development. We performed a developmental time course on wildtype mice at postnatal day 5 (p5), p7, p9 and p14 in order to examine normal morphology, expression of water channel marker aquaporin-5 (AQP5), and proliferation levels in developing Brunner's glands. Our results show that Brunner's gland morphology and cell structure can be

appreciated as early as p5, AQP5 is present as early as p5, and cellular proliferation within the gland is higher at an earlier developmental stage. These results provide a basis for later comparison to other mouse models. Understanding how normal patterns are varied in mice that are genetically altered may shed light on the developmental origins of Brunner's glands and pathological etiologies.

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## Introduction

Brunner's glands are unique to mammalian species and are located in the submucosa of the duodenum, the proximal small intestine adjacent to the pyloric sphincter of the stomach. These glands are composed of secretory glandular epithelium including clusters of serous cells and branching tubules (Collaco et al., 2013).

Secretions from Brunner's glands contain bicarbonate, growth factors, and mucin glycoproteins that lubricate and protect the proximal duodenal mucosa (Krause, 2000).

Bicarbonate secretions from the gland neutralize highly acidic chyme from the stomach as it enters the duodenum. Brunner's glands also synthesize and release glycoproteins, which form a viscous gel that adheres to and lubricates the surface of the proximal duodenum. This mucus barrier protects the duodenal mucosa from acidic and abrasive chyme entering from the stomach (Krause, 2000). Without this gland, highly acidic chyme from the stomach may damage the epithelial cells at the intestinal luminal surface and cause ulceration. Although Brunner's glands serve an important physiological purpose, it is unclear how pathologies, such as Brunner's gland adenomas, arise.

Brunner's gland adenomas occur in both humans and mice that lack *Lrig1*, a negative regulator of epidermal growth factor receptor (EGFR; Powell et al., 2012). EGFR is an ErbB receptor that promotes proliferation of intestinal stem cells, which can lead to tumor growth (Roberts et al., 2002). Tumors arising from Brunner's glands are rare but can result in gastrointestinal bleeding and duodenal obstruction (Gao et al., 2006). Brunner's gland adenomas have been strongly correlated with concurrent *Helicobacter pylori* (*H. pylori*) infections (Kovacevic et al., 2001). *H. pylori* is a

bacterium that causes chronic inflammation in the digestive tract and increases the risk of developing duodenal and gastric ulcers as well as gastric and intestinal cancer (Wroblewski et al., 2011). To understand how disease states of the Brunner's gland arise, we must first elucidate the molecular mechanisms behind Brunner's gland development.

At birth, the small intestine is half its eventual adult length (FitzSimmons et al., 1988). Brunner's glands develop postnatally in both humans and mice, reaching full development by postnatal day 14 (p14; Obuoforibo and Martin, 1977). The gastrointestinal tract begins to form during the third week of fetal life and is derived from the endoderm (Hill, 2017). During embryogenesis, the primitive gut is a tube that can be divided into three segments, termed the foregut, midgut, and hindgut. The foregut is the anterior portion of the primitive gut. The proximal duodenum, stomach, liver, pancreas, and esophagus develop from the foregut, whereas the remainder of the duodenum, ileum, jejunum, and colon arise from the midgut (Hill, 2017). The gastrointestinal tract, including organs such as the stomach, liver, and pancreas, take shape by folding, budding, and rotation of this tube. Different cell types within the gastrointestinal tract form based on the presence of specific signaling proteins which direct the path of cellular differentiation.

In order to understand the etiology of Brunner's gland pathologies, it is vital to study the role of the proteins and stem cells that contribute to Brunner's gland development from the primitive foregut. Identification of these proteins is based on known differentiation pathways of intestinal epithelial cells. Simple columnar epithelial cells that line the intestine are continuously shed and must be replaced by new cells

every three to five days (Umar, 2010). Intestinal epithelial cells are repopulated by highly proliferative stem cells located at the base of intestinal crypts (Figure 1). These stem cells divide and differentiate into different cell types in response to proteins such as *Notch* and *Wnt*. Secretory cells and absorptive cells are two prominent cell types within the small intestine. Secretory cells, which produce secretions in glandular tissue, arise from *Wnt* signaling. Absorptive cells, which are important for nutrient absorption in the villi of the small intestine, arise from *Notch* signaling. Of the secretory cell types in the small intestine, goblet cells, which produce mucins, are most similar in function to the cells of the Brunner's gland (Specian et al., 1991). The differentiation of goblet cells from secretory progenitor cells involves *Spdef* transcription factor



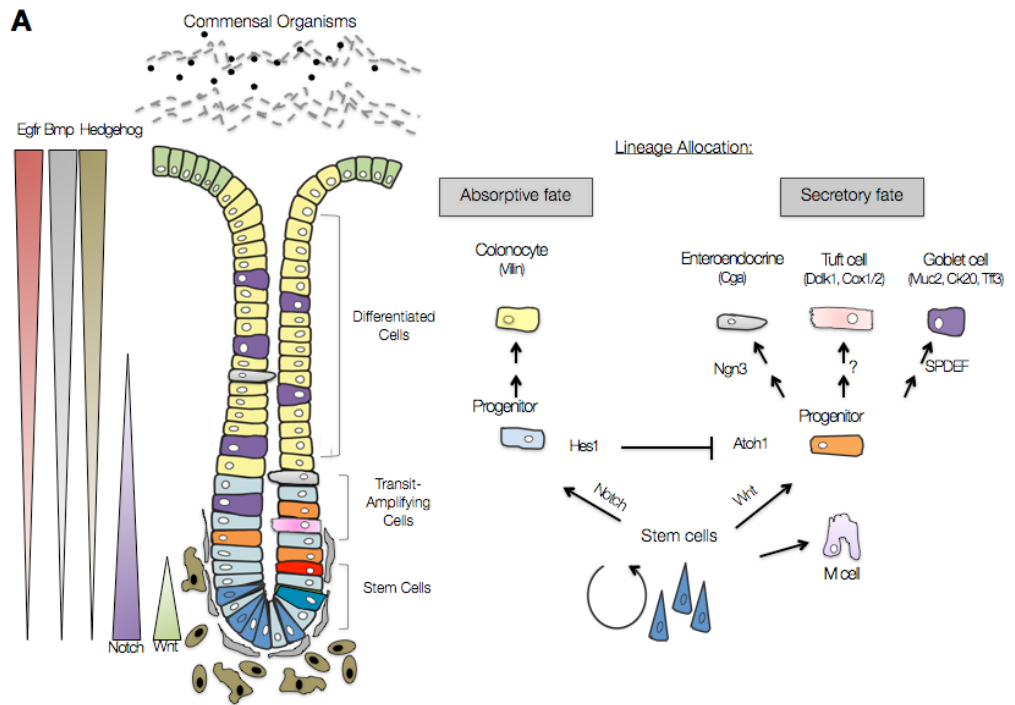


Figure 1. Lineage allocation in intestinal stem cells depends on particular signals early in differentiation. Signaling by *Notch* pushes cells towards an absorptive fate while signaling by *Wnt* pushes cells towards a secretory fate (Powell Lab, University of Oregon, unpublished).

Based on the functional similarity between Brunner's gland cells and secretory intestinal goblet cells, we hypothesized that *Spdef* may be important for the proper formation of Brunner's glands. Previous research indicates that adult Brunner's glands of transgenic mice lacking *Spdef* protein, termed *Spdef* null (*Spdef*<sup>-/-</sup>) mice, have altered morphology and expression of markers such as water channel protein aquaporin-5 (AQP5) (Parappilly et al., in preparation). AQP5 has been identified on the apical and basolateral surfaces of cells throughout the Brunner's glands of adult mice (Parvin et al., 2005). This protein is involved in electrolyte secretion and the osmotic movement of water through the membranes of Brunner's gland cells (Collaco et al., 2013).

These results suggest that *Spdef* is important for normal Brunner's gland development, and that loss of *Spdef* may alter the normal function of the Brunner's

gland. We hypothesize that loss of Spdef alters normal proliferation levels and onset of differentiation markers in the developing gland. In order to identify whether molecules such as Spdef are important for proper formation of Brunner's glands, we needed to establish a baseline for normal development of glands in wildtype (WT) mice. The overall aim of this study was to examine morphology, proliferation levels, and expression of marker AQP5 in the Brunner's glands of WT mice at postnatal day 5 (p5), p7, p9 and p14. This developmental time course will serve as a baseline for later comparison to Brunner's gland development in Spdef null mice.

## Methods

### *Preparation of Tissues*

The laboratory mouse, *mus musculus*, is commonly used to research human disease development and progression. Mice are an advantageous model organism because they reproduce and mature quickly and are genetically similar to humans. This study used wildtype C57B6/6J mice (JAX#000664) that were bred under sterile conditions to minimize gastrointestinal pathologies and were free of the bacteria *H. pylori*.

Mice were euthanized humanely at p5, p7, p9, p14, and adult (>6 weeks) in accordance with Animal Care Services euthanasia protocol. The stomach and duodenum were isolated and washed with a phosphate-buffered saline solution to rinse them of any contaminants. The tissue was then fixed using 4% buffered paraformaldehyde. Paraformaldehyde fixative is used to preserve the tissue from decay by creating protein-crosslinks. If tissue is inadequately fixed it can become brittle and is more likely to be damaged and torn.

After fixation, the specimen was dehydrated in a 70% ethanol solution overnight and cleared with xylenes in preparation for embedding in a paraffin wax block. Embedding is critical for preserving tissue morphology and providing support during sectioning of the tissue. Brunner's glands embedded in paraffin blocks were cut into 5  $\mu\text{m}$  sections using a microtome. These sections were then mounted onto charged glass microscope slides for histological analysis.

## *Histology*

Hematoxylin and eosin (H&E) staining was performed to characterize the morphology of the developing Brunner's gland. H&E is one of the most common staining methods used in histology. Hematoxylin is a positively charged, basic dye that binds to the negatively charged phosphodiester backbone of DNA and stains the nuclei of cells purple. Eosin is a negatively charged, acidic dye that stains basic cellular components, such as the cytoplasm and cellular proteins, pink. H&E staining was used to distinguish between secretory and absorptive cell types in the duodenum in order to identify Brunner's gland tissue.

## *Antibody Staining (Immunofluorescence and Immunohistochemistry)*

Antibody stains were performed on Brunner's gland tissue of WT mice at p5, p7, p9, p14, and adult. Antibody staining utilizes fluorescently tagged antigens to visualize a specific protein of interest within the cell. Slides first undergo antigen retrieval in a 10 mM citrate buffer. Antigen retrieval disrupts protein crosslinks so that antigens in the tissue are exposed and can be bound by primary antibody. The slide is incubated in blocking buffer for one hour to prevent nonspecific binding of the primary antibody. The slide is then incubated with rabbit anti-AQP5 (1:200, Alomone Labs #AQP-500), rat anti-KI67-PE (1:200, eBioscience #12-5698-82), or rat anti-BrdU (1:500, Bio Rad #OBT0030G) overnight. The next day a fluorescently tagged secondary antibody is added to the slides, which binds to the primary antibody. This adds a fluorescent marker to the protein of interest and allows it to be observed under a fluorescent microscope. Lastly, the slide is washed in a phosphate-buffered saline solution containing 4',6-diamidino-2-phenylindole (DAPI). DAPI binds to DNA in the

nucleus and emits a blue fluorescence, which was used to quantify the number of cells in the Brunner's glands.

#### *Ki-67 Immunodetection*

In order to determine normal proliferation patterns in developing Brunner's glands, tissue underwent antibody staining to visualize Ki-67 protein, a cellular marker for proliferation. Ki-67 protein is absent in the G<sub>0</sub>, or resting, phase of the cell cycle and is therefore present only in cells that are actively dividing (Scholzen et al., 2000). The cell cycle is a series of steps that take place as the cell divides to produce two daughter cells. During the first phase of the cell cycle, termed interphase, the cell grows, duplicates its DNA, and accumulates nutrients in preparation for cellular division. Interphase is divided into three stages, termed Growth 1 (G<sub>1</sub>), Synthesis (S), and Growth 2 (G<sub>2</sub>). After interphase, the cell enters its mitotic phase, where chromosomes separate, and then undergoes cytokinesis, the final phase where the cell separates into two new daughter cells. After dividing, the cell leaves the cycle and enters the G<sub>0</sub> resting phase. If a cell is fully differentiated, it will stay in the G<sub>0</sub> phase indefinitely.

#### *BrdU Labeling*

5-Bromo-2'-deoxyuridine (BrdU) is a thymine analogue that was used to detect DNA replication in Brunner's gland cells at various developmental stages which include p5, p7, p9, and p14. BrdU was injected into the abdomen of mice at p1 (10  $\mu$ m, 50  $\mu$ L, Sigma B5002-500MG). When present in the cell, BrdU becomes incorporated into replicating DNA during the S phase of the cell cycle. Due to the semi-conservative nature of DNA replication, BrdU is also incorporated into daughter cells and can therefore be used to lineage trace cells that were dividing at p1. Brunner's glands were

harvested at the various time points and BrdU labeling was visualized using immunofluorescence.

### *Microscopy*

A Nikon Eclipse Ni microscope was used in this study to image Brunner's gland cells using fluorescent microscopy. Specific components of cells were labeled with fluorescent dye during antibody staining. Fluorescent microscopy can be used to image samples that fluoresce, or absorb one wavelength of light and emit another. Fluorescent microscopes radiate the sample with a specific wavelength of light that the sample will absorb. The electrons within the sample become excited to a higher energy level and emit light when they fall to a lower energy state. This releases energy which can be detected as visible light. The microscope filters and measures this emitted light to create an image of the sample.

### *Cell Counting & Statistical Analysis*

BrdU and Ki-67 proliferation values were determined by counting the number of BrdU-labelled and Ki-67 expressing Brunner's gland cells at p5, p7, p9 and p14 (n=3). At least 1,000 cells were counted at each developmental time point. The average BrdU and Ki-67 proliferation values for the sample of three or more mice were graphed for each time point (Figure 6). The total number of cells in the Brunner's gland was determined by counting the number of cells costained with DAPI and AQP5. AQP5 is a Brunner's gland marker, meaning that it is expressed throughout the Brunner's gland and not in the immediately surrounding tissues. Costaining with AQP5 allows for differentiation between Brunner's gland cells and other cells in the duodenum. Next, the number of Brunner's gland cells costained with BrdU and AQP5 or Ki-67 and AQP5

were counted and divided by the total number of cells in the gland. This ratio represents the percentage of proliferating cells in the Brunner's gland.

Statistical analysis was performed on quantified proliferation data to confirm the validity of the experimental results. The standard deviation of BrdU and Ki-67 expression values were calculated for each time point to account for variation between samples. An unpaired t-test was performed to determine whether the difference between the average proliferation values are statistically significant between time points p5 and p7, p7 and p9, then p5 and p14. An unpaired t-test verifies whether a change in proliferation levels between any two time points is significant or is due to random variations in data. This was done by calculating a p-value. A p-value of less than 0.05 indicates that a result is statistically significant, meaning that it is unlikely to have occurred by chance. The lower the p-value, the greater the confidence that a change in proliferation is meaningful.

## Results

My first aim was to characterize the morphology of Brunner's glands during development. To do this, mice were sacrificed at p5, p7, p9, and p14 and tissue containing Brunner's glands was mounted on microscope slides. This tissue underwent H&E staining and images were obtained using light microscopy at 20X magnification. As seen in Figure 2, Brunner's glands can be appreciated as early as p5. The gland increases in size but retains its cell structure and morphology throughout development.

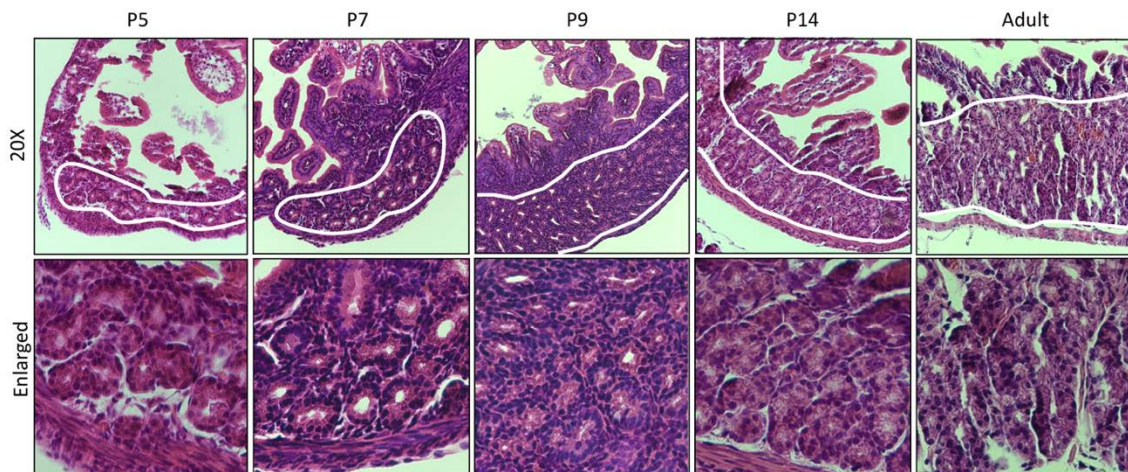


Figure 2. Hematoxylin and eosin staining of wildtype Brunner's glands. The white line indicates separation between Brunner's gland cells and intestinal epithelial components. Presence of Brunner's glands can be appreciated as early as p5. The gland retains its cell structure and morphology throughout development.

My next aim was to examine normal expression patterns of functional marker AQP5 in developing glands. Water channel protein AQP5 has been identified in Brunner's glands of adult, WT mice and is important for transport of electrolytes and osmotic movement of water through the gland (Collaco et al., 2013). In order to establish this, slides were obtained from the same tissue that was used in Figure 2. This tissue underwent antibody staining to determine when expression of AQP5 first appears in Brunner's glands. Images were obtained using a Nikon Eclipse Ni microscope and



edited in Adobe Photoshop. AQP5 protein (green, Figure 3) is present as early as p5 in WT Brunner's glands. Although the size of the gland increases, all Brunner's gland cells are stained with AQP5 regardless of size.

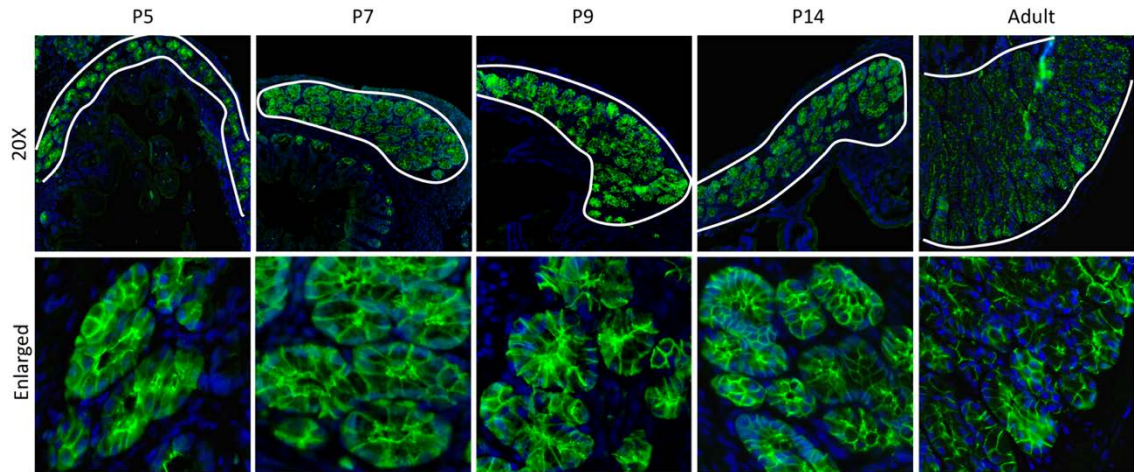


Figure 3. Antibody staining for AQP5 (green) suggests that AQP5 is present as early as p5 in WT Brunner's glands. All Brunner's gland cells express AQP5 at each developmental stage (n=4). DAPI is shown in blue.

Lastly, Ki-67 immunodetection and BrdU-incorporation assays were implemented to determine normal cellular proliferation patterns in the developing wildtype gland. Slides were obtained from the same tissue used in Figures 2 and 3. These mice were injected with BrdU (10  $\mu$ m, 50  $\mu$ L) at p1 before being sacrificed. Slides underwent antibody staining for BrdU or Ki-67 protein, and images were obtained using a Nikon Eclipse Ni microscope and edited in Adobe Photoshop. Ki-67 expression (red, Figure 4) and BrdU-labelling (red, Figure 5) are higher earlier in development and lower later in development.

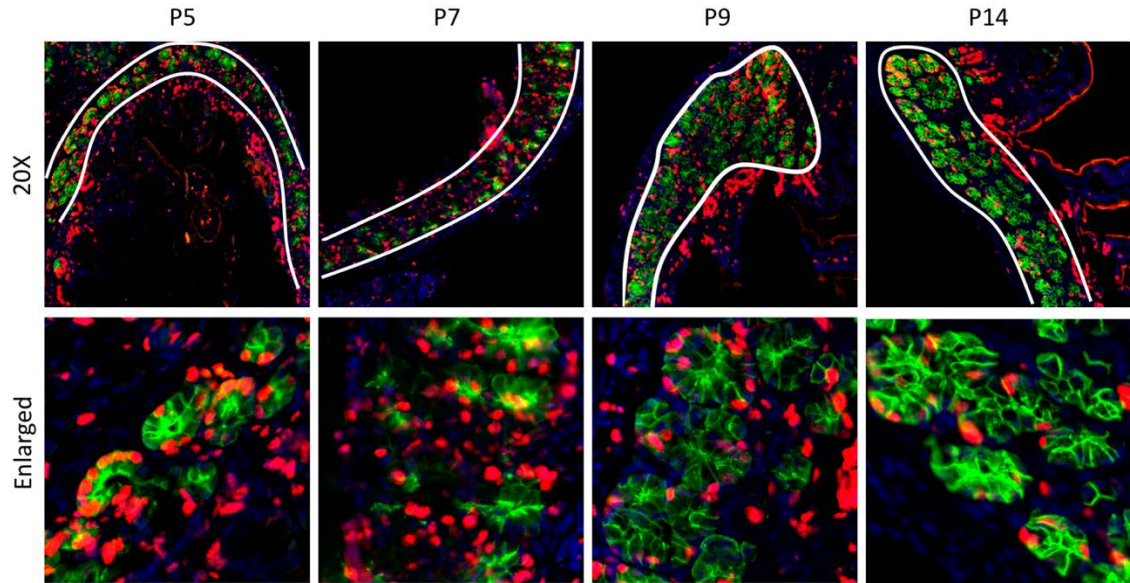


Figure 4. Antibody staining for Ki-67 (red) and AQP5 (green) indicates that Ki-67 expression is higher earlier in development (n=3). DAPI is shown in blue.

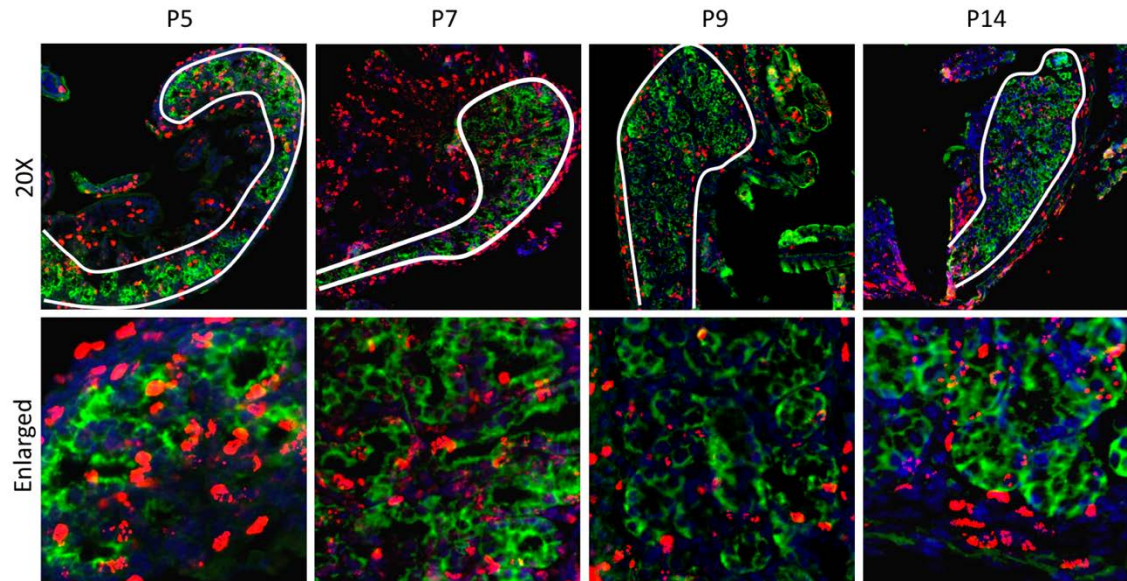


Figure 5. Antibody staining for BrdU (red) and AQP5 (green) indicates that BrdU-labeling is higher earlier in development (n=4). DAPI is shown in blue.

Tissue was co-stained for AQP5 protein, a Brunner's gland marker, in order to quantify the number of Brunner's gland cells on each microscope slide and determine what percentage of these cells express Ki-67 or were labeled with BrdU. This data can be seen in Figure 6.

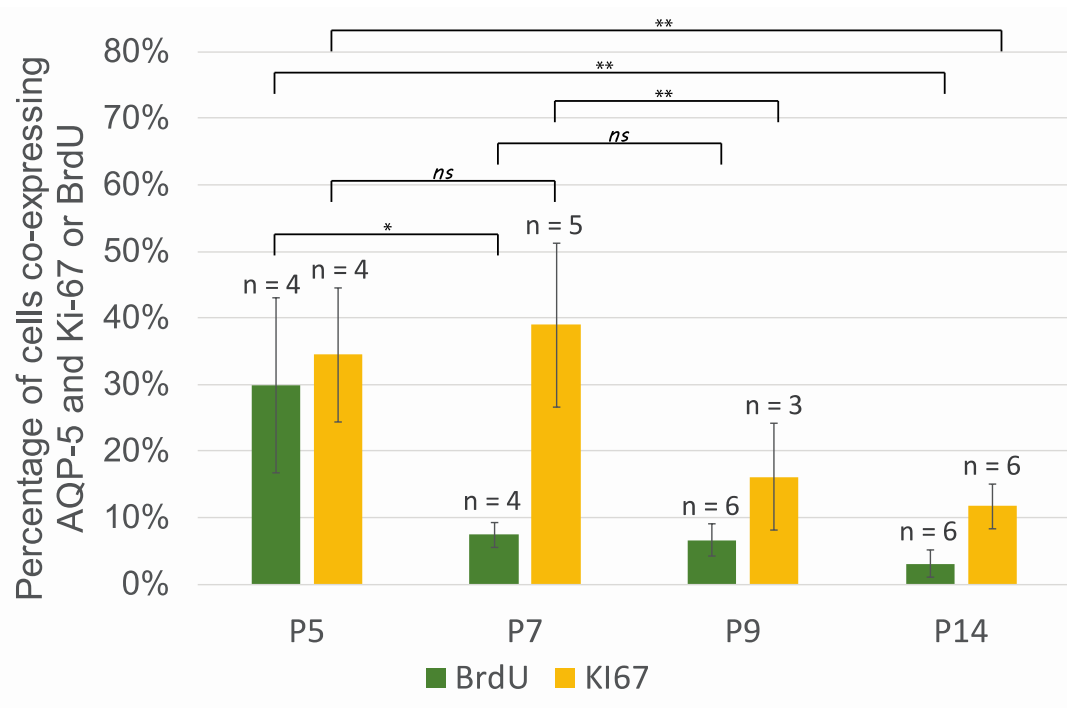


Figure 6. Average BrdU and Ki-67 expression values were graphed at each developmental time point (n=3). Bars indicate statistical significance based on analytical comparison of two time points. These results indicate that Brunner's glands are most proliferative at an earlier developmental stage.

Error bars indicate the standard deviation of each data set. Bars indicate statistical significance based on unpaired t-tests. One star indicates a p-value of less than 0.05, two stars indicate a p-value of less than 0.001, and *ns* indicates no significance between the two time points. Based on this analytical comparison, the difference between average proliferation values of p5 and p14 are significant for both Ki-67 and BrdU assays. We found that the difference in BrdU-labelling of p5 and p7 values is significant but that between p7 and p9 is not significant. We also found that the difference in Ki-67 expression values of p5 and p7 are not significant but that between p7 and p9 is significant.

## Discussion

Research on the function and development of Brunner's gland cells has been largely overlooked, however Brunner gland pathologies such as tumors of the Brunner's gland arise in humans and can cause life-threatening health issues.

The first finding in this study is that typical Brunner's gland morphology and cell structure can be appreciated at p5 in WT mice. Evidence for this was provided through H&E staining of the proximal duodenal tissue in WT mice at p5, p7, p9 and p14. This finding establishes the presence of Brunner's glands. Altered Brunner's gland morphology was observed in adult *Spdef*<sup>-/-</sup> mice. This indicates that *Spdef* protein is important for proper development of the Brunner's glands, and suggests that *Spdef* may impact Brunner's glands function. This first finding can be used as a basis for comparison. We hypothesize that Brunner's glands in *Spdef*<sup>-/-</sup> mice would exhibit altered morphology during development or would not be appreciated until a later time point. This comparison may provide more insight into the role of *Spdef* during development.

The second finding is that AQP5 is present as early as p5 in WT mice. This result may shed light on the role of Brunner's glands during development and help identify factors that are important for proper Brunner's gland development. Since AQP5 protein is essential for the transport of ions and water through Brunner's glands, absent or diminished expression of AQP5 protein may indicate a loss of function in the glands. AQP5 expression is variable in glands of adult, *Spdef*<sup>-/-</sup> mice (Parappilly et al., in preparation). We hypothesize that AQP5 expression will be variable or delayed in glands of *Spdef*<sup>-/-</sup> mice during development. These two findings also indicate that

morphological problems and variable AQP5 expression in *Spdef*<sup>-/-</sup> Brunner's glands do not arise from a developmental delay in the early developmental stages of WT mice. No altered morphology or variable AQP5 expression were observed in any of the WT developmental time points.

The final finding is that Brunner's gland cells are most proliferative at an earlier stage as indicated by Ki-67 and BrdU staining. Assaying Ki-67 proliferation specifies cells that are actively undergoing cellular division at the time point of interest, when mice are sacrificed. Assaying BrdU proliferation specifies cells that were labelled with BrdU at p1 and their daughter cells. Since BrdU injections occur only once at p1, mice sacrificed at a later age will have fewer BrdU labelled cells because they are diluted by new cell growth. Less Ki-67 expression at a later age means fewer cells are actively proliferating in the Brunner's glands.

A developmental time course of Brunner's gland morphology, functional marker expression, and proliferation levels will provide a basis for later comparison in other mouse models, specifically in *Spdef*<sup>-/-</sup> mice. Understanding how normal patterns are varied in mice that are genetically altered may shed light on the developmental origins of the Brunner's glands. Transcription factors such as *Spdef* can push cells toward either a secretory or an absorptive fate through the molecular signaling pathway seen in Figure 1. *Spdef* is important for the differentiation of goblet cells, a secretory cell type in the intestine. Brunner's glands of *Spdef*<sup>-/-</sup> mice may have fewer secretory cells and therefore may exhibit developmental delays that alter the function of the gland. The findings in this study can be used to compare morphology, functional marker expression, and proliferation levels in mice that lack a specific transcription factor that

is important in this signaling pathway. This basis for comparison can also provide insight into how environmental factors such as diet impact Brunner's gland function and development.

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